

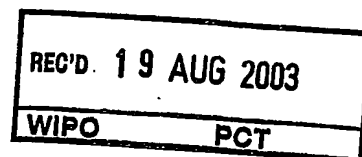
Rec'd PCT/PTO 07 DEC 2004



Europäisches
Patentamt

European
Patent Office

Office européen
des brevets



Bescheinigung

Certificate

Attestation

Die angehefteten Unterla-
gen stimmen mit der
ursprünglich eingereichten
Fassung der auf dem näch-
sten Blatt bezeichneten
europäischen Patentanmel-
dung überein.

The attached documents
are exact copies of the
European patent application
described on the following
page, as originally filed.

Les documents fixés à
cette attestation sont
conformes à la version
initialement déposée de
la demande de brevet
européen spécifiée à la
page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

02077219.0

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk



Anmeldung Nr:
Application no.: 02077219.0
Demande no:

Anmeldetag:
Date of filing: 07.06.02
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

Kylix B.V.
Buntlaan 44
3971 JD Driebergen
PAYS-BAS

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se référer à la description.)

New chemical entities to modulate the activity of exchange proteins directly
activated by camp (Epacs)

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s)
revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C07H/

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of
filing/Etats contractants désignées lors du dépôt:

AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR

07.06.2002

1

(102)

NEW CHEMICAL ENTITIES TO MODULATE THE ACTIVITY OF EXCHANGE
PROTEINS DIRECTLY ACTIVATED BY CAMP (EPACS)

Epacs are a family of guanine nucleotide exchange
5 factors for small GTPases of the Ras family. These proteins
are activated by direct binding of cAMP. By rational design
we have identified and synthesized new chemical entities that
specifically activate Epacs. The new chemical entities
include cAMP analogues with a modified 2'-O-ribose group.

10

Introduction

cAMP, the first identified and well studied second
messenger (1) , plays a role in a wide variety of cellular
processes. Generally, it was assumed that the effects of cAMP
15 are mediated by PKA, the ubiquitously expressed intracellular
receptor for cAMP, although additional cAMP targets have been
described, like the olfactory and pacemaker channels.

Interestingly, recently we and others identified a family of
Rap1 guanine nucleotide exchange factors directly activated
20 by cAMP [Epac1 and Epac2 (also known as cAMP-GEFI and cAMP-
GEFII)] (2-4) . These widely-expressed proteins contain a cAMP
binding pocket that is very similar to the cAMP binding
pocket in the regulatory subunits of PKA, and cAMP is
critically required for exchange activity of Epac1 and Epac2
25 towards the small GTPases Rap1 and Rap2.

Commonly used reagents to activate PKA, like
forskolin, which activates adenylate cyclase, and 8-Br-cAMP,
activate both the PKA- and the Epac mediated signalling
pathways. To circumvent this problem we have searched for a
30 specific stimulus to activate the Epac-Rap1 signalling
pathway. Since Epac differs from PKA in the cAMP binding
domain on at least one critical amino acid, we developed an
Epac specific cAMP analogue, 8CPT-2Me-cAMP, that specifically

binds and activates Epac but not PKA in vitro. Using this analogue in vivo, we found that Rap1 is activated efficiently, whereas PKA-mediated responses are not induced. 8CPT-2Me-cAMP affected neither the activation nor the inactivation of ERK. Instead, we found cAMP-induced ERK activation to be critically dependent on PKA and Ras, whereas Rap1 activity was completely dispensable. The results clearly demonstrate and further strengthen the idea that cAMP-induced Rap1 activation and cAMP regulation of ERK are independent processes.

Results

A cAMP analogue specific for Epac Comparing the amino acid sequences of the cAMP binding domains of Epac with all other cNMP binding domains described in literature, including the cAMP domains of PKA, olfactory and pacemaker channels and the bacterial CAP protein, we noticed that the highly conserved glutamate that makes hydrogen bonding with the 2'-OH of the ribose group of cAMP (5) was absent the cAMP binding domain of Epac1 and in the high affinity cAMP binding domain-B of Epac 2 (Figure 1A). This suggested to us that this 2'-OH group, which is absolutely required for high affinity binding of cAMP to the cAMP binding domain of PKA, might not be required for efficient binding to and activation of Epac. We synthesized and tested a large number of compounds (synthesis by Biolog, modified after Katoaka et al 6 , one of which 8-(4-Chloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate, or 8CPT-2Me-cAMP (Figure 1B), was a very efficient activator of Epac1 in vitro (detailed biochemical analysis of 8CPT-2Me-cAMP will be described elsewhere). Half-maximal activation of Epac1 was observed at 2.2 μ M 8CPT-2Me cAMP compared to 30 μ M for cAMP (Figure 1C). Interestingly, 8CPT-2Me-cAMP binding to Epac1

results in a three-fold higher maximal activity than cAMP (Figure 1C), showing that 8CPT-2Me-cAMP is a much more potent allosteric regulator of Epac1 than cAMP. In contrast, the ability of 8CPT-2Me-cAMP to activate the type I and type II
5 holoenzyme of PKA was greatly impaired compared to cAMP (Figure 1D). These in vitro results indicate that 8CPT-2Me-cAMP may also be a very potent compound to discriminate between the Epac and the PKA signalling pathways in vivo as well. Therefore, 8CPT-2Me-cAMP was tested in NIH3T3-A14-Epac1
10 cells for the activation of Epac, using Rap1 as a read-out, and of PKA, using phosphorylation of the common PKA substrate CREB 29 as a read-out. Importantly, whereas 8-Br-cAMP induced both the activation of Rap1 and the phosphorylation of CREB (Figure 1E, upper panel), 8CPT-2Me-cAMP induced the
15 activation of Rap1 only. Concentration course experiments show that 8CPT-2Me-cAMP already activates Rap1 at a concentration of 10 μ M (Figure 1E, lower panel), but even at a concentration of 100 μ M, it did not induce CREB phosphorylation. Taken together, we conclude that 8CPT-2Me-cAMP is a highly specific and efficient activator of Rap1 and
20 a very useful tool to discriminate between the PKA mediated and the Epac-Rap mediated signalling pathways. In addition we tested a large number of other new chemical entities for their effect on Epac and PKA (Table 1 and 2).
25 Whereas 8CPT-2'O-Me was clearly one of the best specific activators of Epac, most other compounds tested also activated Epac

Experimental Procedures

30 Reagents

Antibodies against phosphorylated CREB (directed against phosphorylated Ser 133) were obtained from Cell Signalling and antibodies against K-Ras/Rap1 were obtained

from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The following inhibitors and stimuli were used at concentrations indicated, unless stated otherwise: H-89 (10 μ M), was obtained from Biomol Research Laboratories Inc.

5 (Plymouth Meeting, PA, USA). Forskolin (40 μ M) was obtained from ICN, 8-Br-cAMP (1 mM) and 8CPT-2Me-cAMP from Biolog Life Science Institute.

Cells, plasmids, transfections

10 NIH3T3-A14 cells stably expressing Epac1 were grown in DMEM with 10% fetal calf serum and 2 μ g/ml, OVCAR3 cells were maintained in RPMI with 10% fetal calf serum.

In vitro activation of cAMP-dependent protein kinase Cyclic AMP dependent protein kinase I and II were
15 reconstituted from isolated subunits and assayed for kinase activity using 70 mM kemptide as substrate as described previously (7).

In vitro measurements of Epac activity

20 *In vitro* GEF assays were performed as described 3,10. More specifically, 600 nM Rap1b loaded with the fluorescent nucleotide mantGDP was incubated in the presence of 100-fold excess GTP and in the absence or presence of 150 nM Epac1 DEP1. Increasing concentrations of cAMP or 8CPT-
25 2ME-cAMP were added and single exponential curves were fit to the data (see Figure 1C two left panels) to calculate reaction rates. Buffer conditions were 50 mM Tris pH7.4, 150 mM NaCl, 5% glycerol, 5 mM DTE, 60 μ M GTP. Reactions were
30 carried out in 96-well plates and measured in a Cary Eclipse from Varian Inc. using the manufacturers software.

Rap1 activation assays

Rap activation assays were performed as described

previously (9-11). Briefly, cells were lysed in lysis buffer containing 10% glycerol, 1% Nonidet P-40, 50 mM Tris-Cl pH7.5, 200 mM NaCl, 2mM MgCl₂, 1 μ M leupeptin, 0.1 μ M aprotinin, 5 mM NaF, 1 mM NaVo₃. Lysates were clarified by centrifugation and incubated with GST-tagged RalGDS-RBD precoupled to glutathione beads to specifically pull down the GTP bound forms of Ras and Rap1, respectively. Samples were incubated for 1hr at 4°C while tumbling. Beads were washed four times in lysis buffer, remaining fluid was removed with an insulin syringe. Proteins were eluted with Laemmli sample buffer and analyzed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and Western blotting. As a control, Ras and Rap1 levels in whole cell lysates were determined.

Legend

Figure 1. Identification of a cAMP analogue specific for Epac.

- a. Alignment of the cAMP binding domains of PKA, Epac, and olfactory- and pacemaker channels.
- b. Structure of 8CPT-2Me-cAMP.
- c. *In vitro* activation of Epac1. Upper panels, Rap1 loaded with fluorescent Mant-GDP and in the presence of 100-fold excess GTP was incubated with Epac1 DEP in the presence of increasing concentrations of either cAMP (left) or 8CPT-2Me-cAMP (right). Lower panels, the reaction rates for cAMP and 8CPT-2Me-cAMP (left) and the corresponding IC₅₀ and *v*_{max} values (right).
- d. *In vitro* PKA activity of either type I holoenzyme (PKAI) or type II holoenzyme (PKAII) at increasing concentrations of either cAMP or 8CPT-2Me-cAMP.
- e. 8CPT 2Me-cAMP activates Rap1 but not PKA in vivo. Upper panel, NIH3T3-A14-Epac1 cells were treated for

15 min with increasing concentrations of 8CPT-2Me-cAMP in duplo. Cells were lysed and equal amounts of cell lysate were incubated with precoupled GST-RalGDSRBD and Rap1 was assayed by immunoblotting with a Rap1 antibody.

- 5 Phosphorylation of CREB in corresponding cell lysates was analyzed using a phospho-specific CREB antibody. Lower panel, as a control, cells were treated with 8-Br-cAMP for 15 min, and cell lysates were analyzed for activation of Rap1 and phosphorylation of CREB.

10 **Table 1:** Structure of new chemical entities

Table 2: Effect new chemical entities on PKA activation and Epac activation in vitro

References

- 15 1. Robison, G. A., Butcher, R. W. & Sutherland, E. W. Cyclic AMP. *Annu Rev Biochem* 37, 149-174. (1968).
2. de Rooij, J. et al. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature* 396, 474-477. (1998).
- 20 3. de Rooij, J. et al. Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs. *J Biol Chem* 275, 20829-20836. (2000).
4. Kawasaki, H. et al. A family of cAMP-binding proteins that directly activate Rap1. *Science* 282, 2275-2279. (1998).
- 25 5. Su, Y. et al. Regulatory subunit of protein kinase A: structure of deletion mutant with cAMP binding domains. *Science* 269, 807-813. (1995).
6. Kataoka, S. et al. Studies on the synthesis of compounds related to adenosine-3', 5'-cyclic phosphate. VI. Synthesis and cardiac effects of N6,N6,2'-O-trialkyl-, N6,2'-O-dialkyl-, and 2'-O-alkyladenosine-3',5'-cyclic phosphates. *Chem Pharm Bull (Tokyo)* 38, 1596-1600. (1990).
- 30 7. Kopperud, R. et al. Formation of inactive cAMP-saturated

holoenzyme of cAMP-dependent protein kinase under physiological conditions. J Biol Chem 277, 13443-13448. (2002).

8. van den Berghe, N., Cool, R. H., Horn, G. & Wittinghofer, A. Biochemical characterization of C3G: an exchange factor that discriminates between Rap1 and Rap2 and is not inhibited by Rap1A(S17N). Oncogene 15, 845-850. (1997).
9. de Rooij, J. & Bos, J. L. Minimal Ras-binding domain of Raf1 can be used as an activation-specific probe for Ras. Oncogene 14, 623-625. (1997).
10. Franke, B., Akkerman, J. W. & Bos, J. L. Rapid Ca²⁺-mediated activation of Rap1 in human platelets. Embo J 16, 252-259. (1997).
11. van Triest, M., de Rooij, J. & Bos, J. L. Measurement of GTP-bound Ras-like GTPases by activation-specific probes. Methods Enzymol 333, 343-348. (2001).

CLAIMS

07.06.2002

(102)

1. Chemicals for modulating the activity of
5 exchange proteins directly activated by cAMP.

2. Chemicals as claimed in claim 1, selected from
the group consisting of:

2'-Deoxyadenosine-3', 5'-cyclic monophosphate;

8-Bromo-2'-deoxy adenosine-3', 5'-cyclic monophosphate;

10 8-(4-Chloro-phenylthio)-2'-deoxyadenosine-3', 5'-cyclic
monophosphate;

8-(4-Chloro-phenylthio)- N⁶-phenyl-2'-deoxyadenosine-3', 5'-
cyclic monophosphate;

2'-O-Methyladenosine -3', 5'-cyclic monophosphate;

15 8-Bromo-2'-O-methyladenosine -3', 5'-cyclic monophosphate;

8-(4-Chloro-phenylthio)-2'-O-methyladenosine-3', 5'-cyclic
monophosphate;

2'-O-Ethyladenosine -3', 5'-cyclic monophosphate;

2'-O-Propyladenosine -3', 5'-cyclic monophosphate;

20 2'-O-n-Butyladenosine -3', 5'-cyclic monophosphate;

2'-O-isoButyladenosine -3', 5'-cyclic monophosphate;

8-Methylamino-2'-O-methyladenosine-3', 5'-cyclic
monophosphate;

25 8-Methylthio-2'-O-methyladenosine-3', 5'-cyclic
monophosphate;

8-(4-Fluoro-phenylthio)-2'-O-methyladenosine-3', 5'-cyclic
monophosphate;

8-(4-Methyl-cumarinyl-7-thio)-2'-O-methyladenosine-3', 5'-
cyclic monophosphate;

30 8-(Naphtyl-2-thio)-2'-O-methyladenosine-3', 5'-cyclic
monophosphate;

8-Phenylthio-2'-O-methyladenosine-3', 5'-cyclic
monophosphate;

- 8-(4-Nitro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic
monophosphate;
- 8-(2-Amino-phenylthio)-2'-O-methyladenosine-3',5'-cyclic
monophosphate;
- 5 8-Benzylthio-2'-O-methyladenosine-3',5'-cyclic
monophosphate;
- 8-n-Hexylthio-2'-O-methyladenosine-3',5'-cyclic
monophosphate;
- 8-Phenylethylamino-2'-O-methyladenosine-3',5'-cyclic
10 monophosphate;
- 8-(4-Methoxy-phenylthio)-2'-O-methyladenosine-3',5'-cyclic
monophosphate;
- 8-Isopropylthio-2'-O-methyladenosine-3',5'-cyclic
monophosphate;
- 15 8-(Benzimidazolyl-2-thio)-2'-O-methyladenosine-3',5'-cyclic
monophosphate;
- 8-(2-Hydroxy-ethylthio)-2'-O-methyladenosine-3',5'-cyclic
monophosphate;
- 8-Ethylthio-2'-O-methyladenosine-3',5'-cyclic monophosphate;
- 20 8-(2-Amino-ethylthio)-2'-O-methyladenosine-3',5'-cyclic
monophosphate;
- 8-(Pyridinyl-2-thio)-2'-O-methyladenosine-3',5'-cyclic
monophosphate;
- 8-(Benzothiazolyl-2-thio)-2'-O-methyladenosine-3',5'-cyclic
25 monophosphate;
- 8-(4-Methyl-phenylthio)-2'-O-methyladenosine-3',5'-cyclic
monophosphate;
- 8-(3-Methoxy-phenylthio)-2'-O-methyladenosine-3',5'-cyclic
monophosphate;
- 30 8-(4-Isopropyl-phenylthio)-2'-O-methyladenosine-3',5'-cyclic
monophosphate;
- 8-(2,3,5,6-Tetrafluoro-phenylthio)-2'-O-methyladenosine-
3',5'-cyclic monophosphate;

8-(4-Hydroxy-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(2,4-Dichloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

5 8-(4-Chloro-phenylthio)-2'-(N,N-dimethyl)-carbamoyl-adenosine-3',5'-cyclic monophosphate;

8-Methoxy-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-Benzyloxy-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-Bromo-2'-O-methyladenosine -3', 5'-cyclic

10 monophosphorothioate, Sp-isomer;

8-(4-Chloro-phenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphorothioate, Sp-isomer;

8-Bromo-2'-deoxyadenosine -3', 5'-cyclic monophosphorothioate, Rp-isomer;

15 8-Bromo-2'-deoxyadenosine -3', 5'-cyclic monophosphorothioate, Sp-isomer;

8-(4-Chloro-phenylthio)-2'-deoxyadenosine-3', 5'-cyclic monophosphorothioate, Rp-isomer;

8-(4-Chloro-phenylthio)-2'-deoxyadenosine-3', 5'-cyclic

20 monophosphorothioate, Sp-isomer;

8-(4-Chloro-phenylthio)-inosine-3', 5'-cyclic monophosphate;

8-Cyclohexylamino-2'-deoxyadenosine-3', 5'-cyclic monophosphate;

25 3. Synthesis of chemicals as claimed in any one of claims 1 and 2 by any suitable method.

4. Use of chemicals as claimed in any one of claims 1 and 2 for a biological purpose, including, but not exclusive, the treatment of cultured cells, animals or human beings.

30 5. Use of chemicals as claimed in any one of claims 1 and 2 for a biological purpose, including, but not exclusive, the treatment of cultured cells, animals or human beings to modulate the activity of exchange proteins

directly activated by cAMP.

Fig. 1A

07.06.2002

(102)

PKAR1a-A	F G E L A L I Y G T - - - - - P R A A T V K
PKAR1b-A	F G E L A L I Y G T - - - - - P R A A T V K
PKAR1a-B	F G E I A L L M N R - - - - - P R A A T V V
PKAR1b-B	F G E I A L L L N R - - - - - P R A A T V V
PKARIIa-A	F G E L A L M Y N T - - - - - P R A A T I V
PKARIIb-A	F G E L A L M Y N T - - - - - P R A A T I T
PKARIIa-B	F G E L A L V T N K - - - - - P R A A S A Y
PKARIIb-B	F G E L A L V T N K - - - - - P R A A S A H
Epac1	F G Q L A L V N D A - - - - - P R A A T I I
Epac2-B	F G K L A L V N D A - - - - - P R A A S I V
D.M.Epac-B	F G K L A L I N D A - - - - - P R A A T I V
Cel.Epac-B	F G K L A L V N D L - - - - - P R A A T I V
Epac2-A	F G E S - I L D N T - - - - - P R H A T I V
Olfactory	F G E I S I L N I K G S K M G N - R R T A N I R
Pacemaker	F G E M V H L Y A K P G K S N A D V R A L T Y C

Fig. 1B

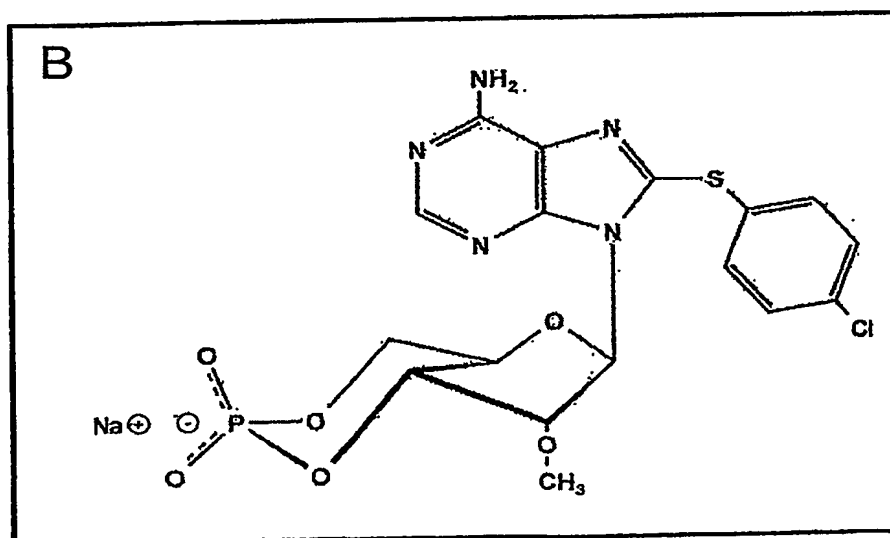


Fig. 1C

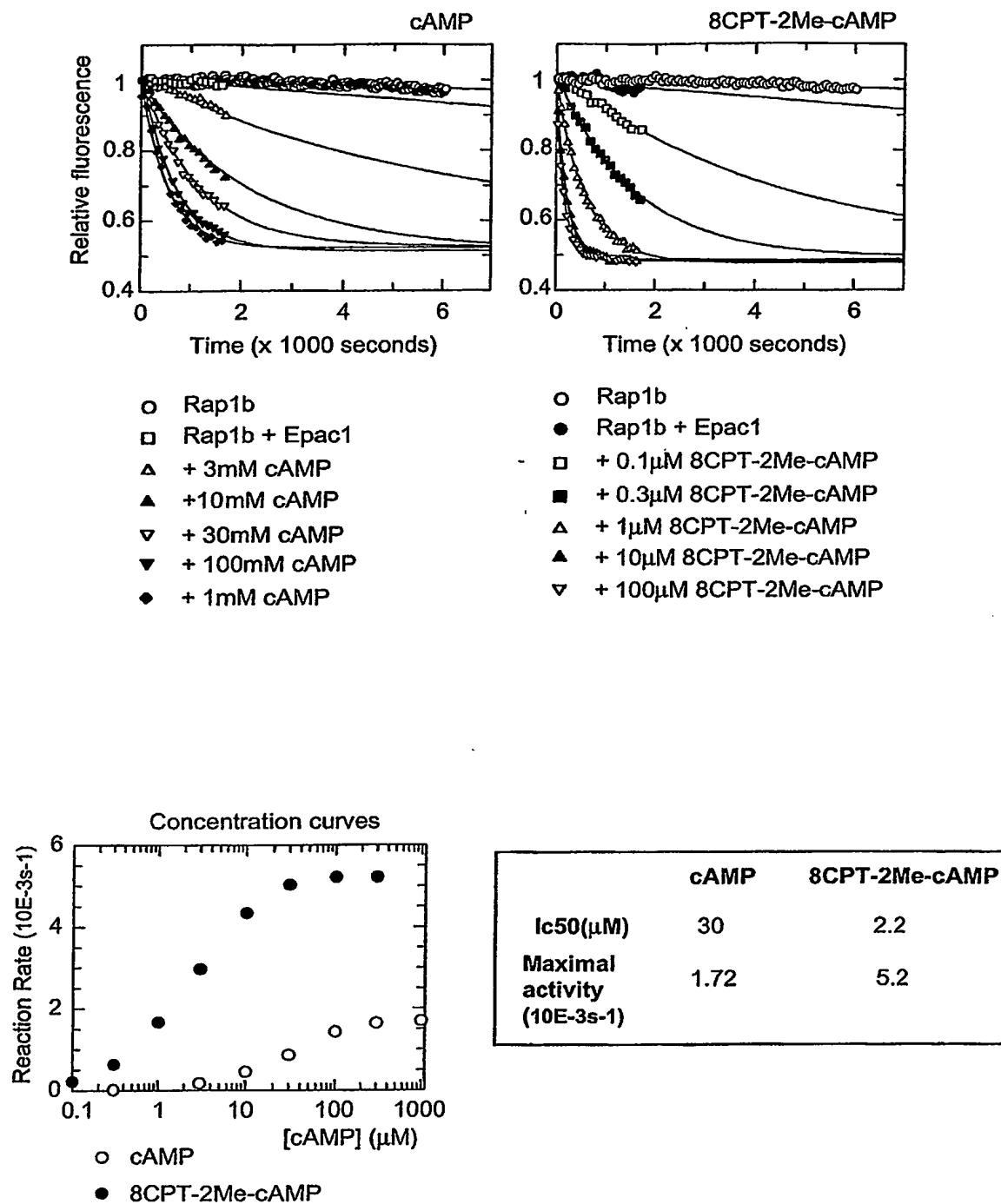


Fig. 1D

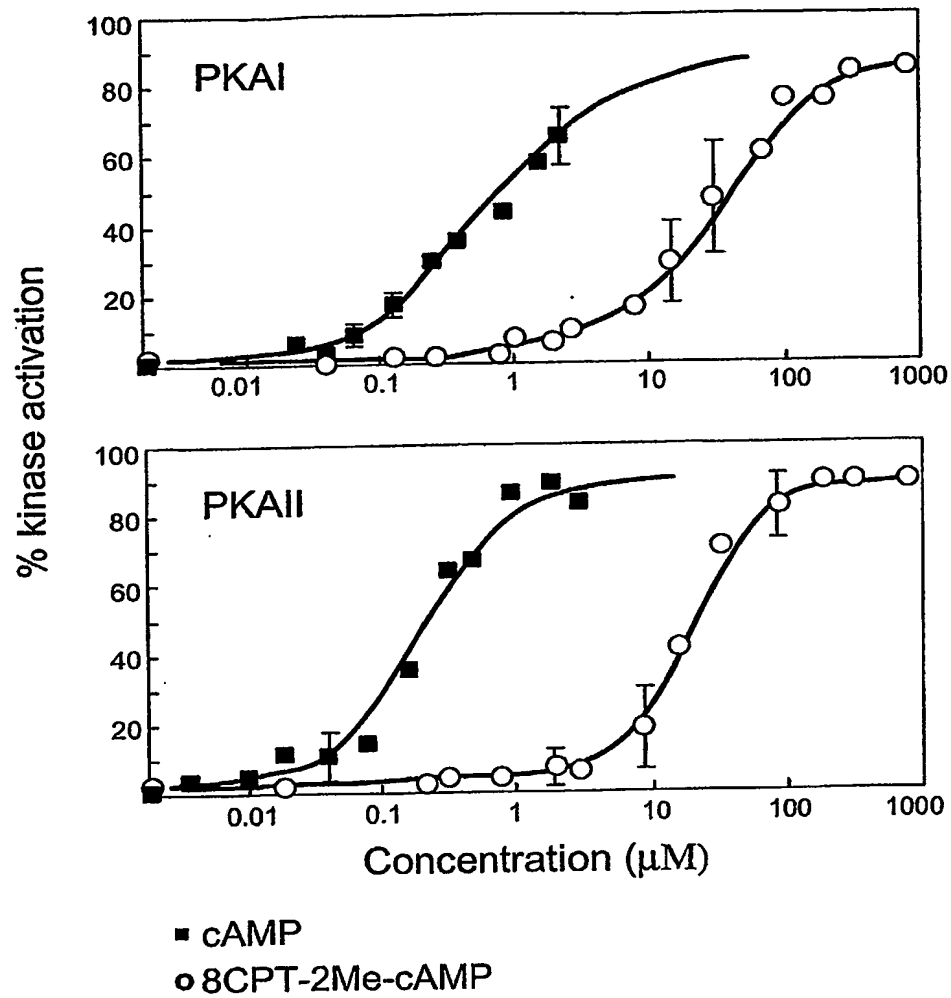


Fig. 1E

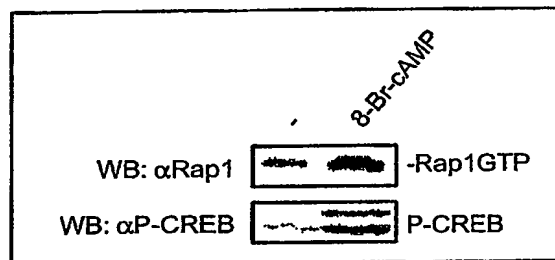
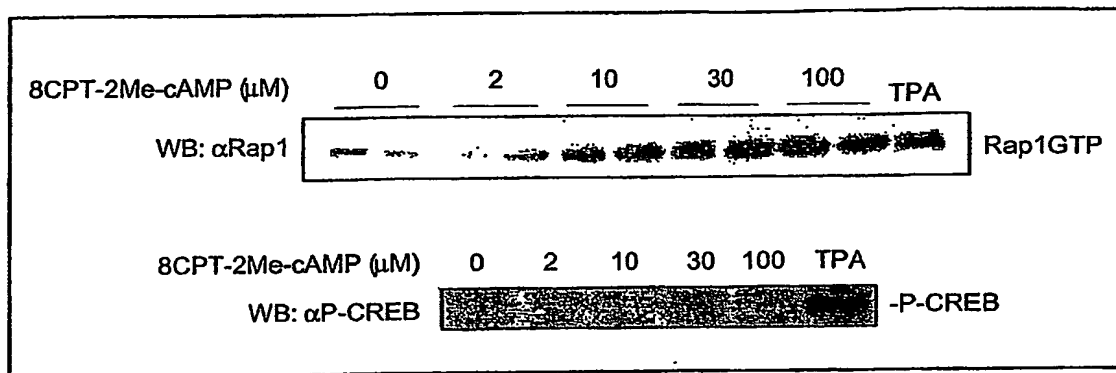
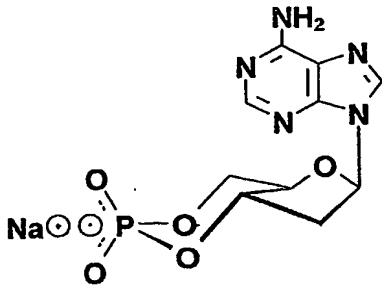
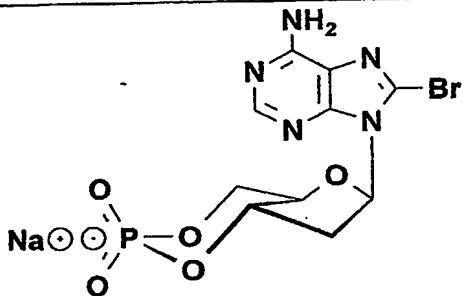
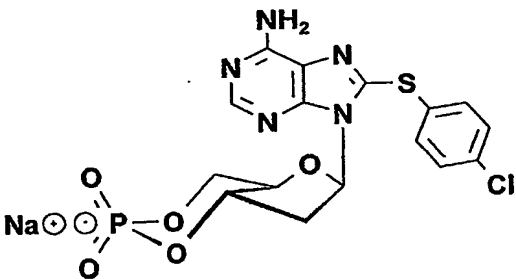
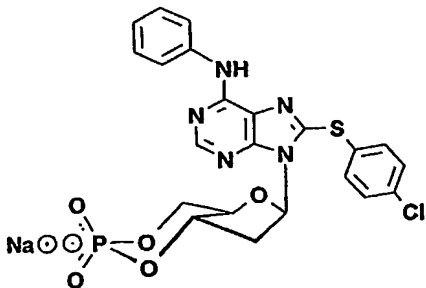
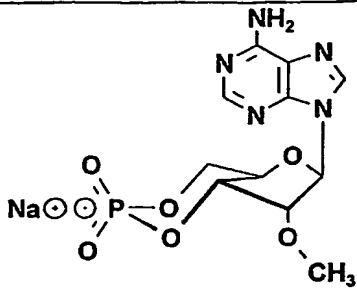
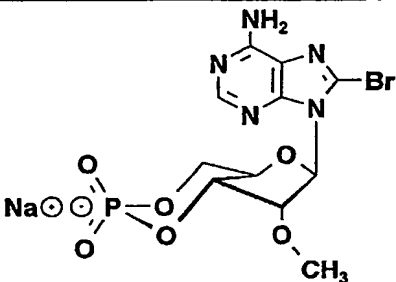
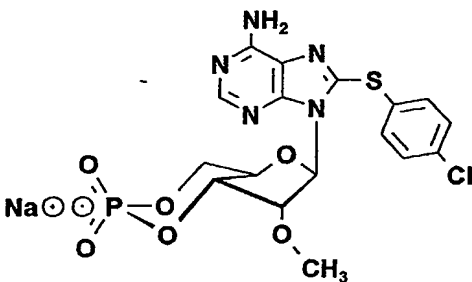
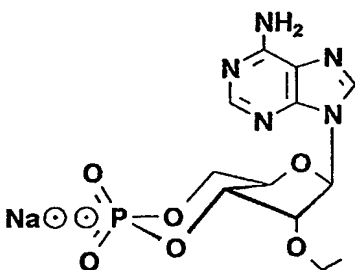
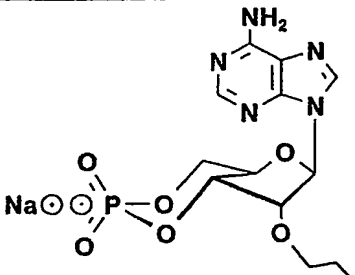
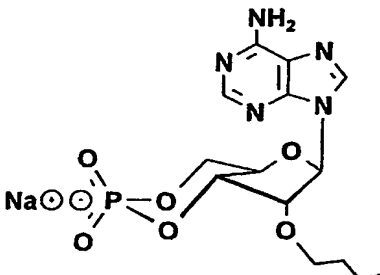
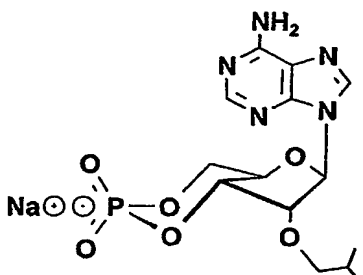
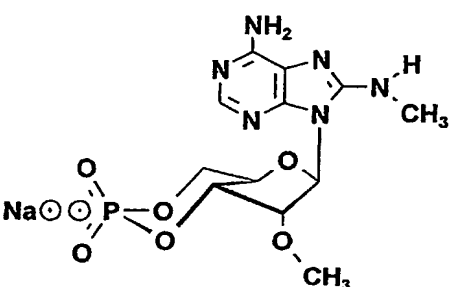
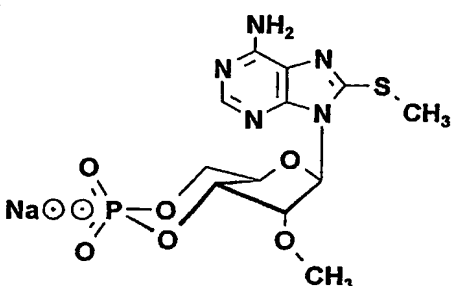
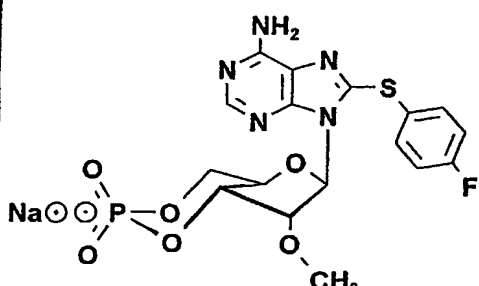
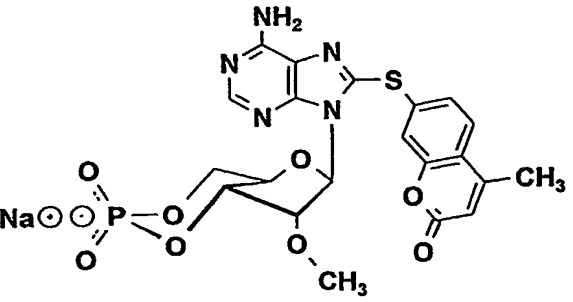
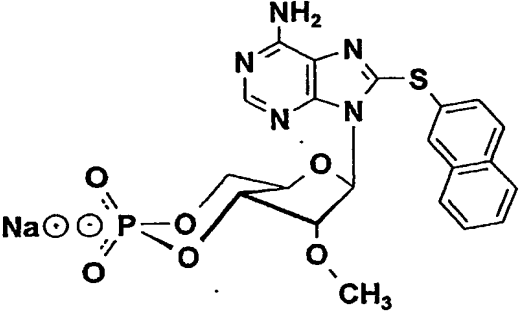
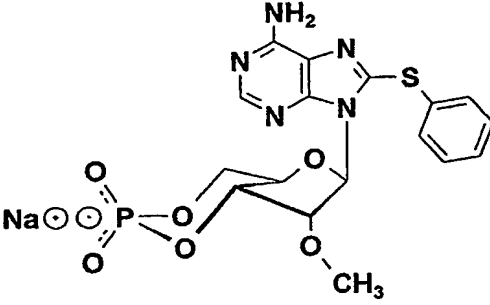
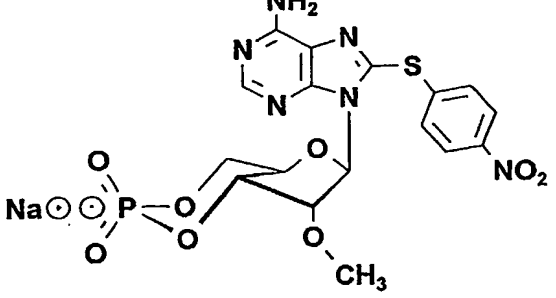


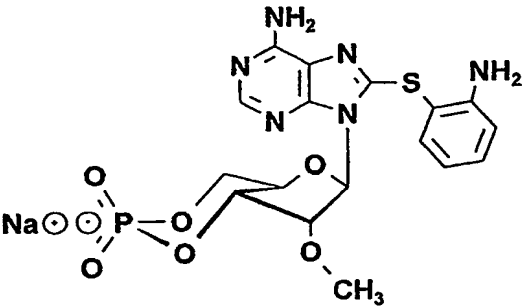
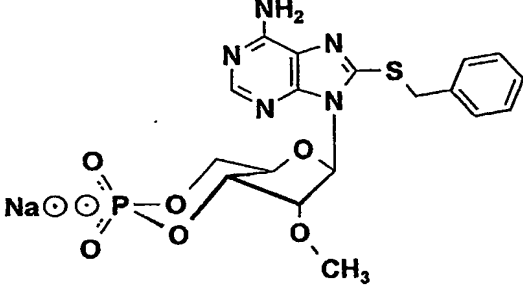
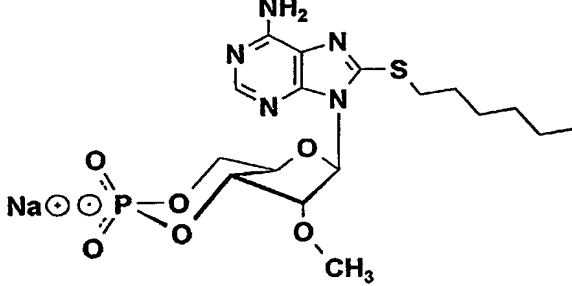
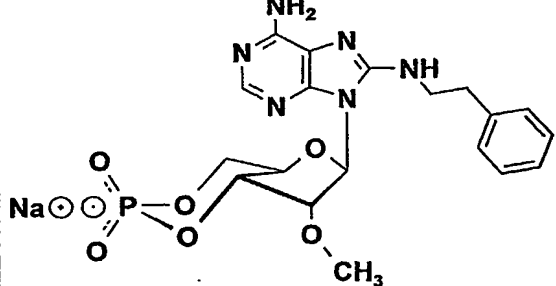
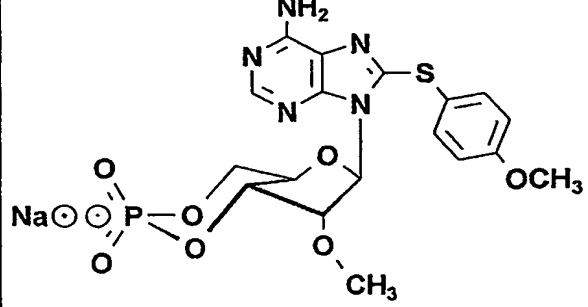
TABLE 1: EPAC-selective cyclic nucleotide analogs

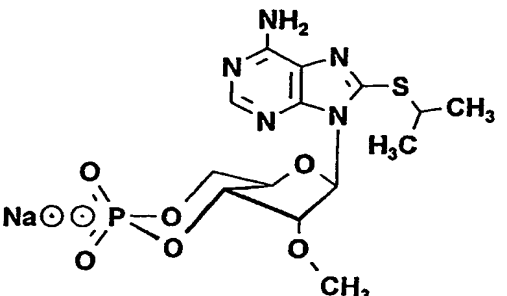
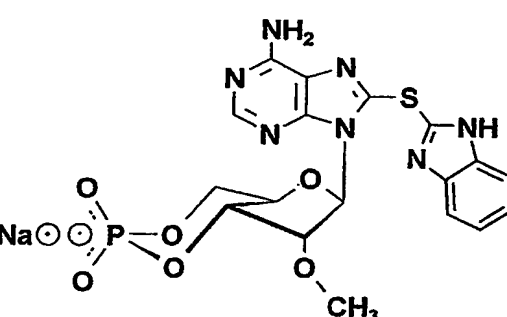
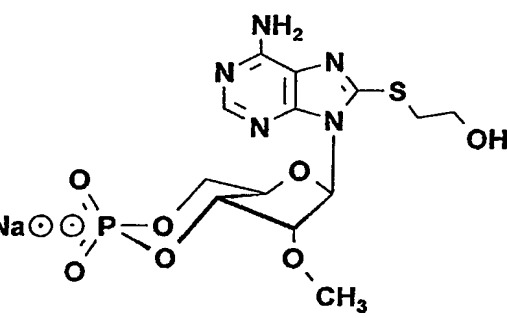
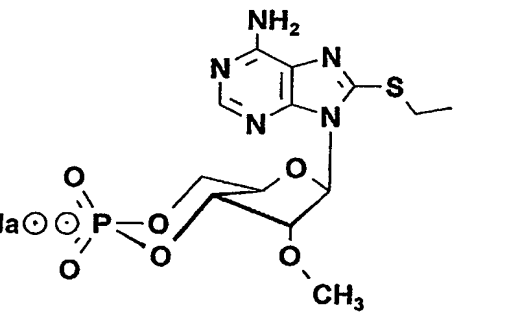
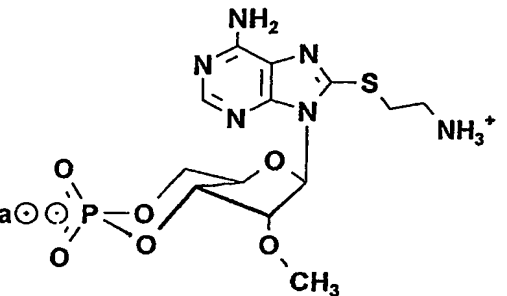
Compound name	Name	Chemical structure	activity
2'-dcAMP 2'-Deoxyadenosine-3', 5'-cyclic monophosphate	I-001		+
8-Br-2'-dcAMP 8-Bromo-2'-deoxy adenosine-3', 5'-cyclic monophosphate	I-002		+
8-pCPT-2'-dcAMP 8-(4-Chloro-phenylthio)-2'-deoxyadenosine-3', 5'-cyclic monophosphate	I-003		+
8-pCPT-6-Phe-2'-dcAMP 8-(4-Chloro-phenylthio)- N ⁶ -phenyl-2'-deoxyadenosine-3', 5'-cyclic monophosphate	I-004		?

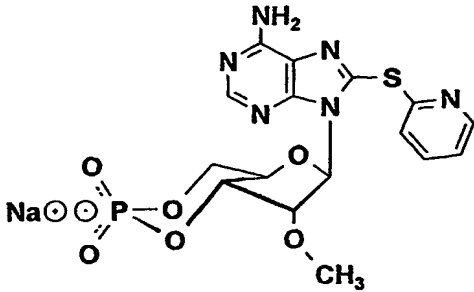
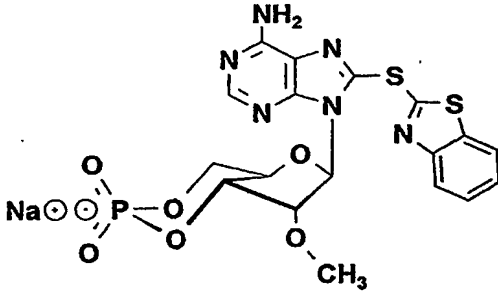
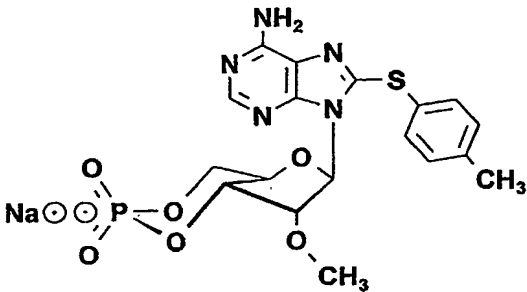
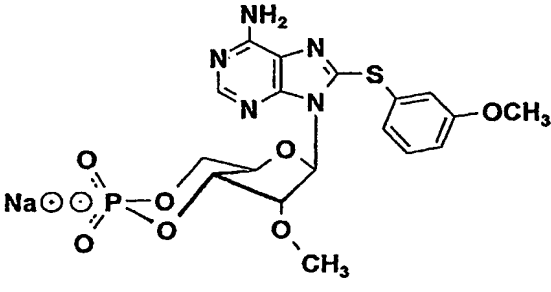
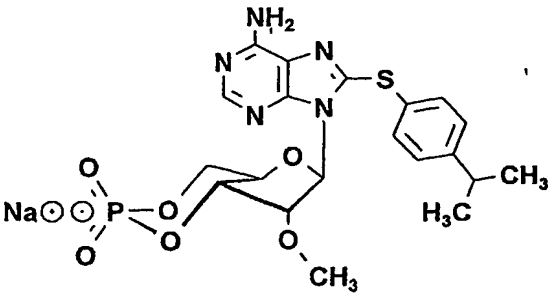
2'-O-Me-cAMP 2'-O-Methyladenosine -3', 5'-cyclic monophosphate	I-005		?
8-Br-2'-O-Me-cAMP 8-Bromo-2'-O-methyladenosine -3', 5'-cyclic monophosphate	I-006		++
8-pCPT-2'-O-Me-cAMP 8-(4-Chlorophenylthio)-2'-O-methyladenosine -3', 5'-cyclic monophosphate	I-007		+++
2'-O-Et-cAMP 2'-O-Ethyladenosine -3', 5'-cyclic monophosphate	I-008		+
2'-O-Pr-cAMP 2'-O-Propyladenosine -3', 5'-cyclic monophosphate	I-009		+

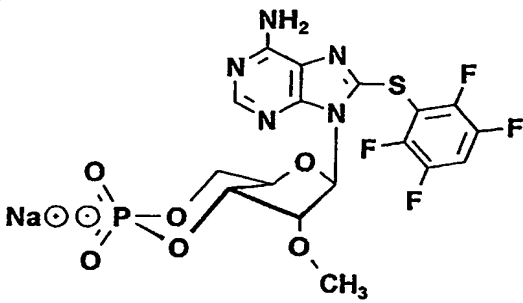
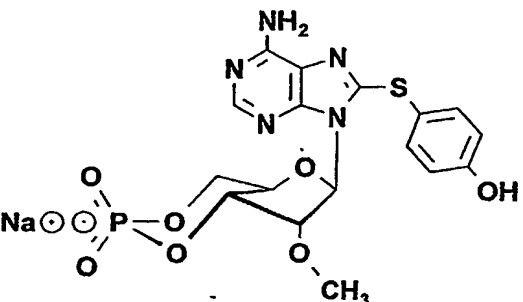
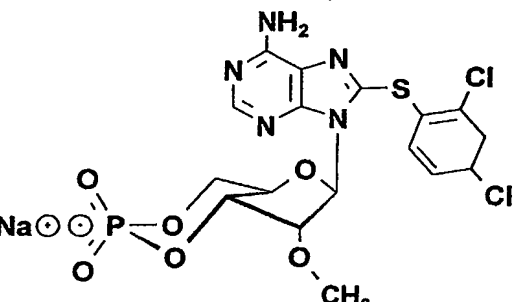
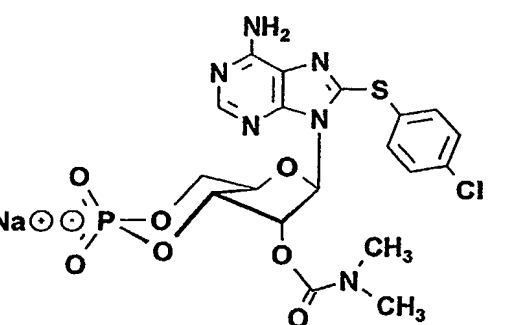
<p>2'-O-Bu-cAMP</p> <p>2'-O-n-Butyladenosine - 3', 5'-cyclic monophosphate</p>	<p>I-010</p>		<p>+</p>
<p>2'-O-iBu-cAMP</p> <p>2'-O-isoButyladenosine - 3', 5'-cyclic monophosphate</p>	<p>I-011</p>		<p>-/+</p>
<p>8-MA-2'-O-Me-cAMP</p> <p>8-Methylamino-2'-O-methyladenosine-3', 5'-cyclic monophosphate</p>	<p>I-100</p>		<p>-/+</p>
<p>8-MT-2'-O-Me-cAMP</p> <p>8-Methylthio-2'-O-methyladenosine-3', 5'-cyclic monophosphate</p>	<p>I-101</p>		<p>+</p>
<p>8-pFPT-2'-O-Me-cAMP</p> <p>8-(4-Fluorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate</p>	<p>I-102</p>		<p>+++</p>

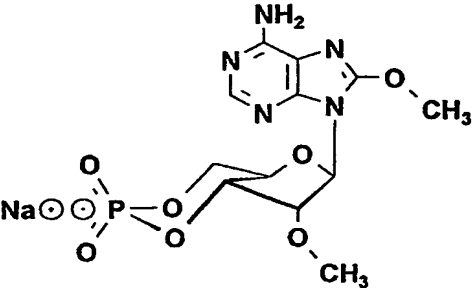
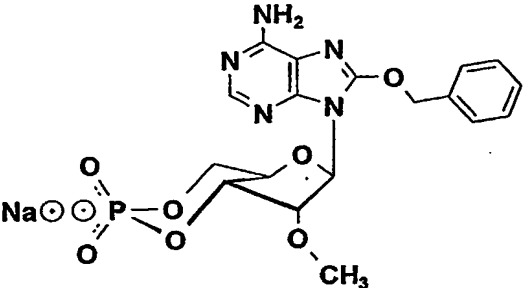
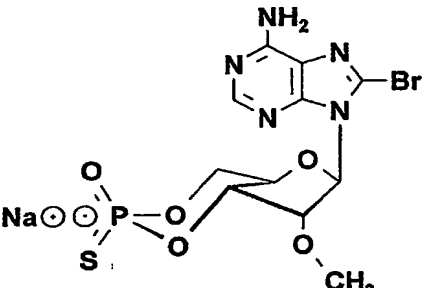
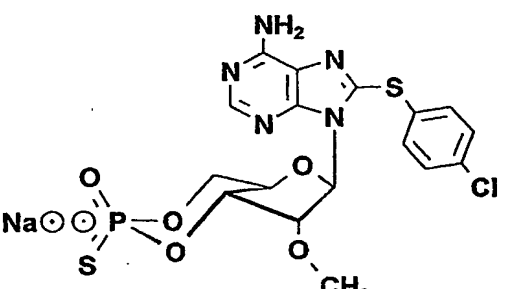
8-MCT-2'-O-Me-cAMP 8-(4-Methyl- cumarinyl-7- thio)-2'-O- methyladenosine- 3',5'-cyclic monophosphate	I-103		-/+
8-NT-2'-O-Me-cAMP 8-(Naphthyl-2- thio)-2'-O- methyladenosine- 3',5'-cyclic monophosphate	I-104		++
8-PT-2'-O-Me-cAMP 8-Phenylthio-2'- O- methyladenosine- 3',5'-cyclic monophosphate	I-105		++
8-pNPT-2'-O-Me-cAMP 8-(4-Nitro- phenylthio)-2'-O- methyladenosine- 3',5'-cyclic monophosphate	I-106		++

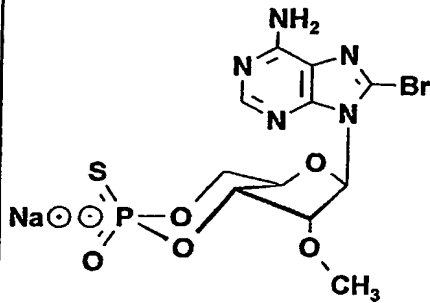
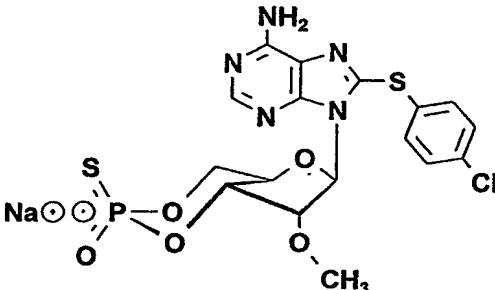
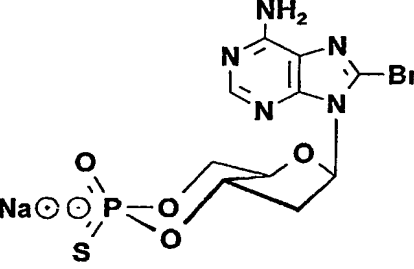
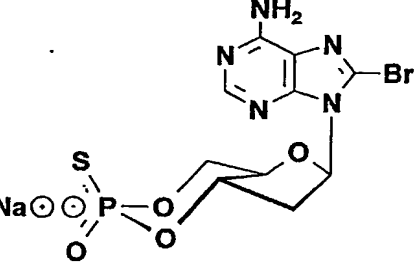
8-<i>o</i>APT-2'-O-Me-cAMP 8-(2-Amino-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	I-107		+
8-BnT-2'-O-Me-cAMP 8-Benzylthio-2'-O-methyladenosine-3',5'-cyclic monophosphate	I-108		+
8-HT-2'-O-Me-cAMP 8-n-Hexylthio-2'-O-methyladenosine-3',5'-cyclic monophosphate	I-109		++
8-PhEA-2'-O-Me-cAMP 8-Phenylethylamin o-2'-O-methyladenosine-3',5'-cyclic monophosphate	I-111		-/+
8-<i>p</i>MeOPT-2'-O-Me-cAMP 8-(4-Methoxy-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	I-112		+++

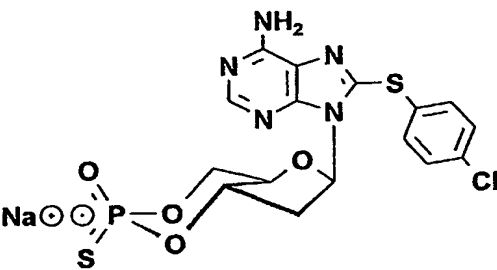
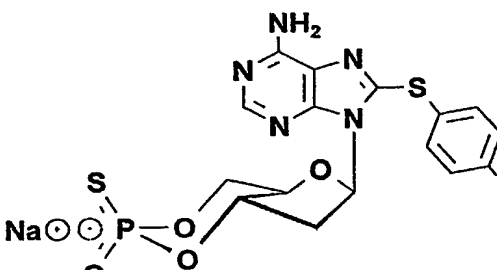
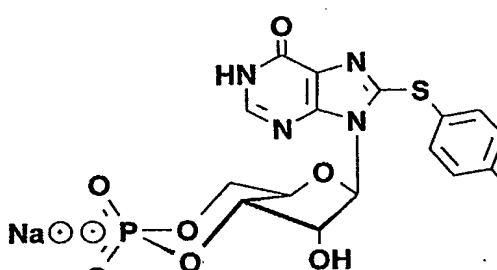
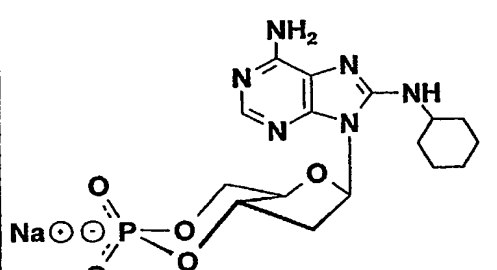
8-IPT-2'-O-Me-cAMP 8-Isopropylthio-2'-O-methyladenosine-3',5'-cyclic monophosphate	I-113		+
8-BIT-2'-O-Me-cAMP 8-(Benzimidazolyl-2-thio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	I-115		+
8-HET-2'-O-Me-cAMP 8-(2-Hydroxyethylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	I-116		+
8-ET-2'-O-Me-cAMP 8-Ethylthio-2'-O-methyladenosine-3',5'-cyclic monophosphate	I-117		+
8-AET-2'-O-Me-cAMP 8-(2-Aminoethylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	I-118		-/+

8-PyT-2'-O-Me-cAMP 8-(Pyridinyl-2-thio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	I-119		++
8-BTT-2'-O-Me-cAMP 8-(Benzothiazolyl-2-thio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	I-120		+
8-pMPT-2'-O-Me-cAMP 8-(4-Methylphenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	I-121		+++
8-mMeOPT-2'-O-Me-cAMP 8-(3-Methoxyphenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	I-122		+
8-pIPPT-2'-O-Me-cAMP 8-(4-Isopropylphenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	I-123		++

<p>8-TFPT-2'-O-Me-cAMP</p> <p>8-(2,3,5,6-Tetrafluorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate</p>	<p>I-124</p>		<p>+</p>
<p>8-pHPT-2'-O-Me-cAMP</p> <p>8-(4-Hydroxyphenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate</p>	<p>I-125</p>		<p>+++</p>
<p>8-DCPT-2'-O-Me-cAMP</p> <p>8-(2,4-Dichlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate</p>	<p>I-126</p>		<p>+</p>
<p>8-pCPT-2'-DMC-cAMP</p> <p>8-(4-Chlorophenylthio)-2'-(N,N-dimethyl)-carbamoyl-adenosine-3',5'-cyclic monophosphate</p>	<p>I-127</p>		<p>-</p>

<p>8-MeO-2'-O-Me-cAMP</p> <p>8-Methoxy-2'-O-methyladenosine-3',5'-cyclic monophosphate</p>	<p>I-131</p>		<p>N.G.</p>
<p>8-BnO-2'-O-Me-cAMP</p> <p>8-Benzyloxy-2'-O-methyladenosine-3',5'-cyclic monophosphate</p>	<p>I-132</p>		<p>N.G.</p>
<p>Rp-8-Br-2'-O-Me-cAMPS</p> <p>8-Bromo-2'-O-methyladenosine-3',5'-cyclic monophosphorothioate, Rp-isomer</p>	<p>I-129</p>		<p>-/+</p>
<p>Rp-8-pCPT-2'-O-Me-cAMPS</p> <p>8-(4-Chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphorothioate, Rp-isomer</p>	<p>I-130</p>		<p>-/+</p>

<p>Sp-8-Br-2'-O-Me-cAMPS</p> <p>8-Bromo-2'-O-methyladenosine -3', 5'-cyclic monophosphorot hioate, Sp-isomer</p>	I-133		?
<p>Sp-8-pCPT-2'-O-Me-cAMPS</p> <p>8-(4-Chloro-phenylthio)-2'-O-methyladenosine -3', 5'-cyclic monophosphorot hioate, Sp-isomer</p>	I-134		?
<p>Rp-8-Br-2'-dcAMPS</p> <p>8-Bromo-2'-deoxyadenosine -3', 5'-cyclic monophosphorot hioate, Rp-isomer</p>	I-135		?
<p>Sp-8-Br-2'-dcAMPS</p> <p>8-Bromo-2'-deoxyadenosine -3', 5'-cyclic monophosphorot hioate, Sp-isomer</p>	I-136		?

<p>Rp-8-<i>p</i>CPT-2'-dcAMPS</p> <p>8-(4-Chlorophenylthio)-2'-deoxyadenosine-3', 5'-cyclic monophosphorothioate, Rp-isomer</p>	<p>I-137</p>		<p>?</p>
<p>Sp-8-<i>p</i>CPT-2'-dcAMPS</p> <p>8-(4-Chlorophenylthio)-2'-deoxyadenosine-3', 5'-cyclic monophosphorothioate, Sp-isomer</p>	<p>I-138</p>		<p>?</p>
<p>8-<i>p</i>CPT-cIMP</p> <p>8-(4-Chlorophenylthio)-inosine-3', 5'-cyclic monophosphate</p>	<p>I-139</p>		<p>?</p>
<p>8-cHA-2'-dcAMP</p> <p>8-Cyclohexylamino-2'-deoxyadenosine-3', 5'-cyclic monophosphate</p>	<p>I-140</p>		<p>?</p>

relative affinity	cAMP=1			relative activity of Epac *
	PKAI	PKAII	Epac	
I-001				+
I-002				+
I-003	0.001	0.001	0.2	+
I-004			0.5	ND
I-005	0.005	0.005	0.12	ND
I-006	0.0015	0.003	0.9	++
I-007	0.009	0.003	4.6	+++
I-008	0.0043		0.05	+
I-009	0.001		0.03	+
I-010	0.0008		0.03	+
I-011				±
I-100	0.0014	0.0001	<0.5	±
I-101	0.012	0.0006	1.3	+
I-102	0.02	0.0004	4.5	+++
I-103	0.03	0.0005	1.8	±
I-104	0.03	0.001	2.3	++
I-105	0.02	0.0005	3.6	++
I-106	0.02	0.0002	1.2	++
I-107	0.004	0.0001	1.3	+
I-108	0.04	0.0007	1.3	+
I-109	0.03	0.0008	1.2	++
I-111			0.08	±
I-112	0.025	0.0006	6.5	+++
I-113	0.028	0.0004	0.7	+
I-115			0.09	+
I-116			1.2	+
I-117			1.8	+
I-118			0.34	±
I-119			0.8	++
I-120			0.17	+
I-121			5.1	+++
I-122			2	+
I-123			4.8	++
I-124			4.7	+
I-125			6.4	+++
I-126			3.1	+
I-127			<0.002	-
I-129 (Rp-006)			0.05	±
I-130 (Rp-007)			0.06	±
I-131			0.36	+
I-132			0.18	+
I-133				+
I-134				+

I-135				+
I-136				+
I-137				+
I-138				+
I-139				+
I-140				+

* relative activity of Epac in vitro in the exchange reaction towards Rap

- +++ activity comparable to 007
- ++ activity between 007 and cAMP
- +
- ± activity less than cAMP

TABLE 2